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Specific In Vitro Association Between the Hepatitis C Viral Genome and Core Protein

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Little is known about the molecular interactions required for hepatitis C virion assembly. The 5′ noncoding region (5′NCR) of the RNA genome is highly conserved and has extensive secondary structure. The highly basic core protein is rich in arginine and lysine residues. We postulate that a specific interaction between these structures may be important for virion assembly. Using an RNA gel mobility shift assay, a specific interaction has been demonstrated between the RNA of the 5′NCR and recombinant core protein. Proteins from other regions of the virus do not interact with the viral RNA. The interaction is inhibited competitively by unlabelled sense polarity RNA, but antisense 5′NCR RNA and nonspecific RNAs compete only at much higher concentrations. These data suggest that there is a specific interaction between the 5′NCR of the hepatitis C virus (HCV) genome and HCV core protein. This interaction may be important for the specific encapsidation of the viral genome during HCV replication. ***J. Med. Virol. 59:131–134, 1999.***

KEY WORDS: RNA gel shift assay; virion assembly; viral encapsidation

Hepatitis C virus (HCV) is an important human pathogen responsible for frequent morbidity and mortality from chronic liver disease and hepatocellular carcinoma. It is estimated that at least 1% of the world's population is infected chronically with this virus [Alter, 1995]. To date, a highly effective treatment strategy has not been developed to eradicate this virus. Research in this field has been hampered by the lack of a practical animal of infection, because only humans and chimpanzees are susceptible, and the difficulties experienced in establishing a reproducible, efficient tissue culture system for HCV propagation.

HCV is a positive sense, single-stranded RNA virus of approximately 9.5 kb length. The genome contains a single large open reading frame that encodes a polyprotein of slightly more than 3,000 amino acids (the molecular virology of HCV has recently been reviewed in Clarke, 1997). The polyprotein is processed posttranslationally to yield the structural (core and envelope) and nonstructural proteins of the virus. The coding sequence is flanked by 5′- and 3′-noncoding regions. Genomic sequence analysis suggests that HCV is related to both the pestivirus and flavivirus genera and likely represents the prototype of a novel genus within the *Flaviviridae* family.

The 5′-noncoding region (5′NCR) of HCV is highly conserved among viral isolates [Bukh et al., 1992] and is predicted to contain extensive secondary structure. The viral genome lacks a methylated cap structure and the 5′NCR has been shown to contain an internal ribosome entry site (IRES) [Tsukiyama-Kohara et al., 1992] that may allow cap-independent expression of the viral genome in a manner analogous to that elucidated in the picornaviruses [Jackson et al., 1990].

The core protein is located at the amino terminus of the HCV polyprotein and is likely to form the viral nucleocapsid. The 191-amino acid protein is highly basic in nature and has a hydrophobic carboxy-terminal region. The core protein has been shown to associate with both RNA and ribosomes in vitro [Santolini et al., 1994]. As the building block of the nucleocapsid, the HCV core protein would be predicted to be capable of specific association with the viral genome as well as multimerization. In fact, HCV core protein multimerization has been demonstrated and the domain responsible for multimerization has been localized to the hydrophilic amino-terminal two-thirds of the protein [Matsumoto et al., 1996; Nolandt et al., 1997]. Additionally, the core protein has been shown to interact with one of the viral envelope proteins [Lo et al., 1996].

There is a growing recognition of the importance of the specific interaction of regions of the viral genome

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and viral structural proteins for the assembly of a large number of viruses. RNA viruses including retroviruses [Aronoff and Linial, 1991; Dupraz and Spahr, 1992; Gelfand et al., 1993; Berkowitz and Goff, 1994], alphaviruses [Forsell et al., 1995], picornaviruses [Ansardi et al., 1994; Nugent and Kirkegaard, 1995], rotaviruses [Mattion et al., 1992], alfalfa mosaic virus [Reusken et al., 1994], and others have been demonstrated to have some type of RNA encapsidation signal that mediates association of the viral genome with a viral structural protein. Additionally, hepatitis B virus, a DNA virus that replicates via reverse transcription of an RNA pregenome, specifically encapsidates its pregenome due to the interaction between an RNA encapsidation signal and viral core protein [Junker-Niepmann et al., 1990]. Typically, a short segment of RNA with extensive secondary structure (frequently a stem-loop structure) in a noncoding region of the viral genome interacts with a viral nucleocapsid protein to allow efficient packaging of the viral genome.

We hypothesize that the HCV core protein interacts specifically with the HCV RNA genome and that this interaction is important for virion assembly. An RNA gel mobility shift assay was developed to study the interaction between HCV genomic RNA and recombinant viral proteins [Konarska and Sharp, 1986; Furuya and Lai, 1993].

Recombinant HCV proteins were kindly provided by Dr. J. Kim (Genelabs, Redwood City, Ca.). Briefly, HCV sequences were amplified by polymerase chain reaction (PCR) and cloned into the pGEX plasmid to yield glutathione-*S*-transferase fusion proteins, which were expressed in *Escherichia coli.* Additionally, cDNA corresponding to the first 120 amino acids of HCV core protein was cloned into the pET plasmid to yield the discrete (nonfusion) NC360 protein.

Plasmid pI47 contains the 5′NCR sequence of HCV in pBluescript SK+ (Stratagene). Radiolabelled RNA transcribed in vitro corresponding to the 5′NCR was synthesized using T7 RNA polymerase (Promega) in the presence of [α-^{32}P] UTP (Amersham) after linearization of pI47 with BamHI.

RNA gel mobility shift assays were carried out in a volume of 15 ml in the presence of recombinant HCV protein, 20,000 cpm (0.4–1.0 ng) of [^{32}P] RNA and binding buffer containing 10 mM Hepes (pH 7.6), 3 mM $MgCl_2$, 40 mM KCl, 5% glycerol, 2.5 mM DTT, and 20 U of RNase inhibitor. Following incubation at 26°C for 30 min, the binding reactions were subjected to electrophoresis on a vertical nondenaturing polyacrylamide gel (Mini Protean, BioRad). A 4% acrylamide gel [(acrylamide/methylene bisacrylamide ratio 60:1), 0.5× TBE (45 mM Tris-OH, 45 mM boric Acid, 1 mM EDTA)] in 0.5× TBE was preelectropheresed for 1 hr at 80 V at room temperature. After loading the samples, electrophoresis was carried out for 2 hr under the same conditions, gels were dried and exposed to Xomat film (Kodak) at –80°C.

Twenty nanograms of recombinant pGEX fusion proteins corresponding to the amino half of NS3 (33U); carboxyl half of NS3 (409-1-1); and most of NS4 (5-1-1) failed to shift the radiolabelled RNA probe corresponding to the 5′NCR (data not shown). In contrast, NC450, a pGEX fusion protein corresponding to the amino-terminal 80% of HCV core protein partially shifted the RNA probe (data not shown), whereas NC360, a discrete HCV core protein corresponding to the amino terminal two-thirds, almost shifted fully the RNA probe at the same concentration.

Increasing concentrations of NC360 resulted in a dose-related increase in the intensity of the shifted radiolabelled 5′NCR RNA band (Fig. 1). Any concentration more than 12 ng of NC360 in the 15-μl binding mixture resulted in a nearly complete shift of the RNA band. The specificity of the RNA gel mobility shift as-

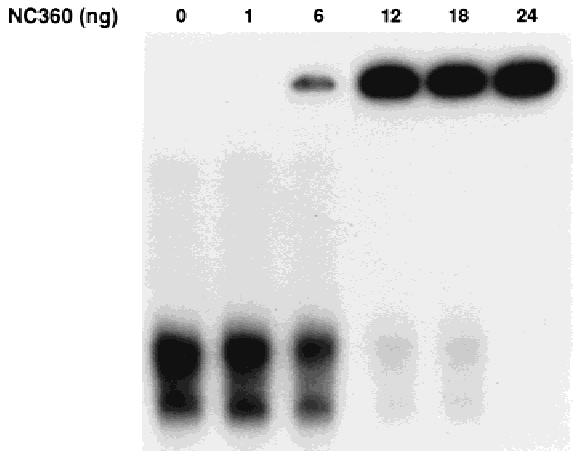

Fig. 1. Effect of hepatitis C virus (HCV) core protein concentration on HCV 5′-noncoding RNA gel mobility shift. Radiolabelled HCV 5′-noncoding region RNA (20,000 cpm) was incubated with 1, 6, 12, 18, or 24 ng of NC360 core protein in a total volume of 15 μl prior to polyacrylamide gel electrophoresis and autoradiography. The first lane contains RNA with no protein. A dose-related increase in RNA gel mobility shift is seen, plateauing above 12 ng protein.

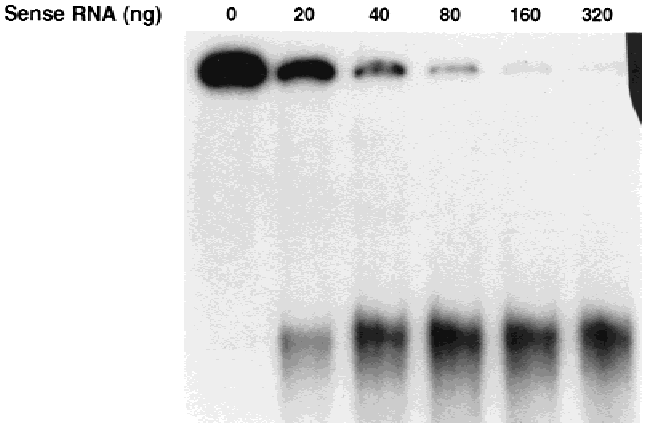

Fig. 2. Competition of hepatitis C virus (HCV) 5′-noncoding RNA gel mobility shift by unlabelled HCV 5′-noncoding RNA. Radiolabelled HCV 5′-noncoding region RNA (20,000 cpm) was incubated with 12 ng NC360 protein in a total volume of 15μl in the presence of 20, 40, 80, 160, or 320 ng unlabelled HCV 5′-noncoding region RNA. The first lane contains no inhibitor. A dose-related inhibition of RNA gel mobility shift is observed.

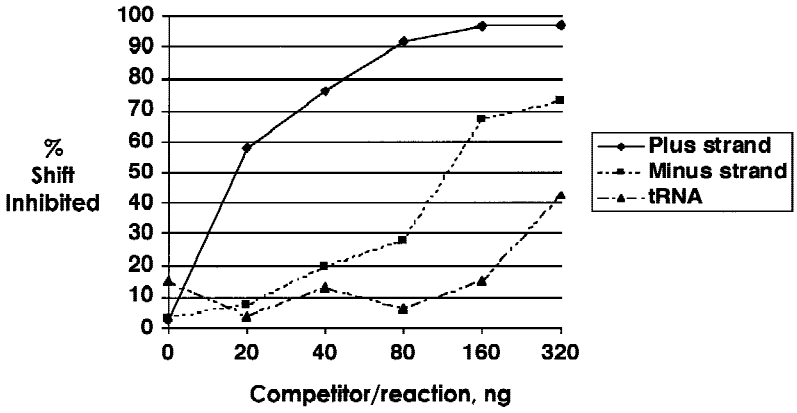

Fig. 3. Competition of hepatitis C virus (HCV) 5′-noncoding RNA gel mobility shift by unlabelled RNAs. RNA gel mobility shift autoradiograms were subject to densitometry and the percentage shift inhibition of radiolabelled HCV 5′-noncoding region RNA was plotted against the concentration of competitor in the reaction. Unlabelled 5′-noncoding region RNA inhibited the RNA gel mobility shift assay at a much lower concentration than unlabelled minus strand RNA or tRNA, demonstrating the specificity of the reaction.

say was demonstrated by the addition of increasing concentrations of unlabelled RNA competitors in the binding mixture. With unlabelled 5′NCR RNA, there was dose-related inhibition of the RNA mobility gel shift induced by 12 ng NC360 (Fig. 2). With 80 ng or more of unlabelled 5′NCR RNA in the binding mixture, the gel mobility shift was almost completely inhibited. In contrast, the addition of unlabelled RNA complementary to the 5′NCR or yeast tRNA only inhibited the gel mobility shift at much higher concentrations and the inhibition was never complete. These results are presented graphically in Figure 3.

In summary, using an RNA gel shift assay, a specific interaction was demonstrated between the core protein of HCV and the 5′NCR of the RNA genome of the virus. The RNA-binding domain of the HCV core protein resides in amino terminal two-thirds of the protein. The carboxyl one-third of the protein is highly hydrophobic and is believed to play an important role in localization of the protein to the endoplasmic reticulum [Lo et al., 1995]. Over 90% of the basic amino acids in the core protein are found within the first 120 residues corresponding to NC360, so it is not unexpected that RNA-binding is resident in this region. Interestingly, truncated NC360 protein shifts 5′NCR RNA more efficiently than NC450. This finding may relate to the fact that inclusion of the carboxyl region of the protein renders it less soluble.

The 5′NCR is highly conserved between viral isolates and has a complex, high degree of secondary structure. Although we have not elucidated fully the RNA domain(s) responsible for the interaction with HCV core protein, the presence of extensive secondary structure is typical of protein-binding RNA sequences.

The HCV core protein has been demonstrated to bind RNA by the Northwestern procedure [Santolini et al., 1994], although the phenomenon was not specific to HCV RNA sequences. In the current study, the interaction between HCV core protein and radiolabelled 5′NCR RNA was competed only partially by high concentrations of complementary RNA or tRNA. This finding suggests that the association is relatively specific, although the possibility of additional lower affinity nonspecific RNA binding by HCV core protein cannot be excluded.

The RNA band shift demonstrated in our studies generally results in the RNA probe not entering the gel. This phenomenon persisted despite a wide range of buffer conditions, electrophoresis conditions, and temperature conditions tested (data not shown). It has been shown previously, using a yeast two-hybrid system and confirmed with an in vitro protein-protein blotting assay, that the HCV core protein binds itself [Matsumoto et al., 1996]. The domain responsible for multimerization has been localized to amino acids 1–115, which is included in NC360. It is conceivable, but remains to be proven, that the phenomena of core protein–RNA interaction and core protein multimerization are interrelated and important for the assembly of viral nucleocapsids. If so, then the complexes not entering the gel may represent 5′NCR RNA associated with multimers of HCV core protein.

A specific interaction was demonstrated between the 5′NCR of the HCV RNA genome and the viral core protein that may be important for HCV virion assembly. This may represent a novel target for antiviral strategies against this important virus.

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